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## A Fungal Glycosphingolipid Directly Activates Natural Killer T Cells and Rapidly Induces Airways Disease

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### Abstract

*Aspergillus fumigatus* is a saprophytic fungus that is ubiquitous in the environment and commonly associated with allergic sensitization and severe asthma in humans. Although *A. fumigatus* is recognized by multiple microbial pattern recognition receptors, we identified and synthesized an *A. fumigatus* glycosphingolipid, asperamide B, that directly activated invariant natural killer T (iNKT) cells *in vitro* in a CD1d-restricted, MyD88- and dectin-1-independent fashion. Moreover, asperamide B, when loaded into CD1d, directly stained, and was sufficient to activate, iNKT cells. *In vivo*, asperamide B rapidly induced airway hyperreactivity, a cardinal feature of asthma, by activating pulmonary iNKT cells in an IL-33-ST2-dependent fashion. Asperamide B is thus the first fungal glycolipid found to directly activate iNKT cells. These results extend the range of microorganisms that can be directly detected by iNKT cells to the Kingdom of Fungi, and may explain the effectiveness of *A. fumigatus* in causing severe chronic respiratory diseases in humans.

### Keywords

NKT cells; *Aspergillus fumigatus*; glycosphingolipid; IL-33; asperamide B

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**Author Contributions:** L.A.A. conceived and initiated the project, planned and performed experiments, and wrote the manuscript. V.C. performed lipid isolation, identification, and synthesis. Y.J.C. planned and performed experiments including the tetramer studies. H.Y.K. performed experiments and edited the manuscript. Y.T.C. isolated and maintained iNKT cell lines. M.P. maintained iNKT cell lines, assisted with experiments, and provided training in invasive plethysmography. R.D.K. helped plan experiments and edited the manuscript. P.B.S. planned lipid isolation, identification, and synthesis and wrote the manuscript. D.T.U. conceived the idea for the project, planned experiments, shepherded the project along and wrote the manuscript.

## Introduction

Asthma is an inflammatory disease of the airways that affects 300 million individuals worldwide. The inflammation in asthma is frequently driven by Th2 cells and adaptive immunity against environmental aeroallergens. Although the allergen-specific Th2 cells that mediate asthma recognize the protein components of allergens, most environmental allergens contain a complex mixture of lipids and carbohydrates that might function as adjuvants to enhance the process of sensitization. For example, house dust mite contains ligands of TLR4<sup>1</sup> and dectin-2<sup>2</sup>, which activate innate immune cells, including dendritic cells (DCs), airway epithelial cells and macrophages, thereby enhancing the capacity of dust mite to stimulate adaptive Th2 cell sensitization. However, other allergens, such as fungi, may utilize additional immunological mechanisms to enhance sensitization.

The saprophytic fungus *Aspergillus fumigatus* contains several clinically important allergens and is highly associated with allergic asthma. *A. fumigatus* spores or conidia are recovered at very high rates from the environment, from within buildings and homes and in soil, resulting in daily respiratory exposure in most individuals<sup>3, 4</sup>. Although *A. fumigatus* can cause invasive infection, primarily in immunocompromised hosts with defects in neutrophil function, in normal healthy individuals *A. fumigatus* conidia are rapidly cleared by phagocytes after recognition through soluble and cell-associated microbial pattern recognition receptors, including TLR2, TLR4, surfactant proteins A and D, C3, dectin-1 and DC-SIGN<sup>5, 6</sup>. However, in some individuals, allergic sensitization to *A. fumigatus* develops. In patients with asthma, allergic sensitization to *A. fumigatus* is associated with severe disease with reduced lung function<sup>7, 8</sup>. Indeed, a syndrome called severe asthma with fungal sensitization (SAFS), is often associated with *A. fumigatus* colonization of the lung<sup>9</sup>. Rarely, particularly in patients with severe asthma or with cystic fibrosis, a unique hypersensitivity response to *A. fumigatus* develops, known as allergic bronchopulmonary aspergillosis (ABPA), associated with very high levels of serum IgE, eosinophilia, bronchiectasis, pulmonary infiltrates and chronic severe asthma symptoms<sup>10</sup>. The precise mechanisms however, for innate immune recognition of *A. fumigatus* that result in allergic sensitization and asthma are not fully known.

We therefore examined an innate-like pathway that might be activated by *A. fumigatus* involving invariant natural killer T (*i*NKT) cells. *i*NKT cells recognize glycolipid antigens presented by CD1d, rapidly produce large quantities of cytokines, and are thought to play an important role in regulating the development of airway hyperreactivity (AHR), a cardinal feature of asthma<sup>11</sup>, although their role in human asthma is less clear<sup>12</sup>. *i*NKT cells express an invariant T cell receptor (*i*TCR), which is highly conserved evolutionarily and thought to serve as a receptor for pathogen associated molecular patterns. Up to now however, only a few microbes have been shown to express glycolipid antigens that can be directly recognized by the *i*TCR of *i*NKT cells. The high degree of conservation of the *i*NKT cell *i*TCR across many mammalian species suggests that other types of microorganisms, including fungi such as *A. fumigatus*, may also express glycolipids that can be recognized by the *i*TCR of *i*NKT cells.

## Results

### An *A. fumigatus* extract rapidly induces airway hyperreactivity

Intranasal treatment of wildtype BALB/c mice for 3 days with *A. fumigatus* extracts resulted in the development of severe AHR and airway inflammation 24 hrs after the last dose (Fig. 1a). Our protocol was significantly shorter than previous protocols with *A. fumigatus*, in which mice were repeatedly challenged with *A. fumigatus* over 2-4 wks to induce potent Th2-mediated adaptive immunity<sup>13-15</sup>. In our studies, the severe AHR and airway inflammation in response to *A. fumigatus* extract occurred independently of adaptive immunity, in mice deficient for MHC class II (*H2.Ab1-Ea*<sup>-/-</sup>), which lack class II restricted CD4<sup>+</sup> T cells, but which have *i*NKT cells (Fig. 1b, c). The induction of AHR by *A. fumigatus* required IL-4 and IL-13, since it did not occur in *Il4*<sup>-/-</sup>*Il13*<sup>-/-</sup> mice (Fig. 1d). Although *Il4*<sup>-/-</sup>*Il13*<sup>-/-</sup> mice failed to develop AHR, they developed airway neutrophilia (Fig. 1e). mRNA for IL-4 and IL-13 but not IFN- $\gamma$  was increased in the lungs after treatment with *A. fumigatus*, as shown by analysis of total lung RNA by Q-PCR (Fig. 1f). These results suggested that the *A. fumigatus* extract contained glycolipids that activated *i*NKT cells.

### Induction of AHR by *A. fumigatus* requires NKT cells

Indeed, the *A. fumigatus* extract failed to induce AHR in *i*NKT cell-deficient *CD1d1*<sup>-/-</sup> mice, while causing severe AHR in wildtype mice (Fig. 2a). The recruitment of neutrophils to the airway however, was independent of CD1d (Fig. 2b). These data suggest that the *A. fumigatus* extract contains several activities, such that AHR was CD1d and IL-4/IL-13 dependent, while inflammation was CD1d and IL-4/IL-13 independent. The number of *i*NKT cells in the BAL and lung increased after treatment with the *A. fumigatus* extract, as determined by flow cytometry, particularly in the lung in terms of percent of TCR $\beta$ <sup>+</sup> cells and absolute number of *i*NKT cells (Fig. 2c-f). Moreover, *Tcra-J18*<sup>-/-</sup> mice, which like *CD1d1*<sup>-/-</sup> mice lack *i*NKT cells, also failed to develop AHR (Fig. 2g), but this response was reconstituted by adoptive transfer of CD1d-tetramer-sorted wildtype *i*NKT cells (Fig. 2g). To control for the possibility that tetramer sorting could activate the transferred *i*NKT cells, unsorted wildtype splenocytes were used to reconstitute *Tcra-J18*<sup>-/-</sup> mice with *i*NKT cells, which also restored the AHR response to *A. fumigatus* (Supplemental Fig. 1). Combined, these experiments showed that *i*NKT cells are required for the development of the acute AHR in response to the *A. fumigatus* extract.

### *A. fumigatus*-induced AHR requires MyD88 but not Ticam1 or dectin-1

*Myd88*<sup>-/-</sup> mice treated with the *A. fumigatus* extract developed a reduced AHR response, and significantly reduced inflammatory response in the BAL fluid compared to wild-type mice (Fig. 3a, b). Moreover, the *Myd88*<sup>-/-</sup> mice failed to respond when challenged intranasally with only 1 dose (rather than 3 daily doses) of *A. fumigatus* extract and evaluated 24 hrs later, in contrast to wildtype mice, which developed severe AHR and airway inflammation (Fig. 3c, d). These findings suggested that *A. fumigatus* extract contained glycolipids that rapidly induce AHR, but that this response required signaling through TLR receptors or through the IL-1 receptor accessory protein (Il1Rap), a component of the IL-1 and IL-33 receptors. In contrast, the *A. fumigatus*-induced AHR response did not

require Ticam1, since *Lps2/Lps2* mice, which have an early stop codon in the *Ticam1* gene, fully responded to three intranasal doses of *A. fumigatus*, as did wildtype mice (Fig. 3e, f). In addition, the AHR response induced by the *A. fumigatus* extract was independent of dectin-1, since treatment with a dectin-1 specific antibody (500 µg/mouse) did not reduce AHR induced with the *A. fumigatus* extract (Fig. 3g), although the dectin-1 antibody reduced the influx of neutrophils into the BAL fluid by 50% (Fig. 3h), and greatly reduced the *in vivo* induction of IL-12 by the dectin-1 ligand, laminarin ( $\beta$ -1, 3- and  $\beta$ -1, 6 glucans from seaweed *Laminaria digitata*)<sup>16</sup> (Fig.3i).

### A purified *A. fumigatus* lipid activates *i*NKT cells

We next examined *A. fumigatus* extract fractions for their capacity to activate primary *i*NKT cell lines. A fraction that contained most of the activating activity was identified, from which a single lipid compound with a molecular mass of 738 was isolated that migrated as a single spot on high performance thin layer chromatography. Further characterization via single and multidimensional NMR spectroscopy and mass spectrometry (manuscript in preparation), revealed spectral data that matched those of a previously reported glycosphingolipid called asperamide B. Asperamide B had previously been isolated from culture extracts of *Aspergillus niger*, an endophytic fungus isolated from marine brown alga<sup>17</sup>. Importantly, asperamide B, the structure of which is shown in Fig. 4a, was present in significantly higher quantities in extracts from *A. fumigatus* than from *A. niger* (Fig. 4b,  $p < 0.001$  student's t-test).

Coculture of BMDC and primary *i*NKT with purified asperamide B (pAsp-B) induced IL-4 and IFN- $\gamma$  production in a dose dependent manner, with IL-4 reaching statistical significance at the highest dose (Fig. 4c and Supplemental Fig. 2a). Furthermore, a synthetic version of asperamide B (sAsp-B) (manuscript in preparation) also induced IL-4 and IFN- $\gamma$  production in *i*NKT cells in a dose dependent manner (Fig. 4d and Supplemental Fig. 2b). Activation of *i*NKT cells by asperamide B required CD1d, since coculture with BMDC from *CD1d1*<sup>-/-</sup> mice failed to induce IL-4 or IFN- $\gamma$  (Fig. 4e and Supplemental Fig. 2c). Asperamide B most likely directly activated *i*NKT cells, since the induction of IL-4 and IFN- $\gamma$  production by both the purified and synthetic asperamide B was MyD88 and Ticam1 independent, similar to the response observed with the control PBS57 ( $\alpha$ -GalCer analog) (Fig.4e). Although previous studies showed that *Aspergillus* indirectly activated *i*NKT cells by stimulating dectin-1 on APCs and inducing IL-12 production<sup>18</sup>, blockade with an anti-dectin-1 mAb had no effect on *i*NKT cell activation by our *Aspergillus* extract or by sAsp-b (Fig. 4f), although the anti-dectin-1 mAb greatly reduced the induction of IL-12 in BMDC by the dectin-1 ligand, laminarin (Fig. 4g). Furthermore, our *Aspergillus* allergen extract induced only small amounts of IL-12 in BMDC (Fig. 4h), indicating that our *Aspergillus* allergen extract, in contrast to other *Aspergillus* extracts<sup>18</sup>, contained little IL-12-inducing pathogen associated molecular patterns (PAMPs, e.g.  $\beta$ -glucans). However, both the purified and synthetic asperamide B also activated primary human *i*NKT cell lines to produce statistically significant amounts of IL-4 and IL-13 (Fig.4i, j). These *in vitro* results suggested that *A. fumigatus* might drive respiratory disease in mice and in humans in part through direct effects of asperamide B on *i*NKT cells.

### Asperamide B loaded CD1d tetramers bind to *i*NKT cells

We loaded CD1d tetramers with the sAsp-B glycolipid, and found that it stained splenic *i*NKT cells, similar to the staining observed with PBS57 loaded tetramers (known to stain all *i*NKT cells) (Fig. 5a). The percentage of CD4<sup>+</sup> cells and the V $\beta$  distribution of the sAsp-B and PBS57 tetramer<sup>+</sup>*i*NKT cells were also similar, as about 85% of both sAsp-B<sup>+</sup> and PBS57<sup>+</sup>*i*NKT cells were CD4<sup>+</sup>, and about 50% were V $\beta$ 8.1/2<sup>+</sup> (Fig. 5b, c). However, small differences were noted in the number of sAsp-B<sup>+</sup> and PBS57<sup>+</sup>*i*NKT cells that stained with anti-V $\beta$ 7 (13.8% for sAsp-B and 11.5% for PBS57) and anti-V $\beta$ 2 mAb (9.8% of sAsp-B and 8.2% of PBS57). The staining results with sAsp-B and PBS57 loaded CD1d tetramers, including the similar V $\beta$  distributions of the stained cells, were confirmed by staining primary *i*NKT cell lines (Supplemental Fig. 3a–c). Incubation of *i*NKT cell lines simultaneously with both tetramers resulted in staining only with PBS57 loaded tetramers (Supplemental Fig. 3d), suggesting that both tetramers could bind the same *i*TCRs. Moreover, plate bound sAsp-B loaded, but not unloaded, CD1d monomers effectively activated primary *i*NKT cell lines (Fig. 5d). The effect of the plate bound CD1d monomers on the *i*NKT cell lines was amplified when a CD28 activating antibody was added to these cultures (Fig. 5e). These results together indicate that the sAsp-B and PBS57 CD1d tetramers stain similar populations of *i*NKT cells, and that asperamide B can be directly recognized by the *i*TCR of *i*NKT cells.

### Asperamide B induces AHR *in vivo* in wildtype mice

A single dose of purified asperamide B administered to wildtype mice induced AHR within 24 hrs, although the asperamide B was less potent compared to PBS57 (Fig. 5f). Asperamide B also induced airway inflammation with a statistically significant increase in macrophages and a small number of neutrophils that did not reach significance (Fig. 5g). The number of lung *i*NKT cells producing IL-4 alone or IL-4 + IL-13 were significantly increased while the increase in cells producing IL-13 alone did not reach statistical significance (Fig. 5h). These numbers were calculated from data of the total number of *i*NKT cells in the lung and the fraction of *i*NKT cells producing IL-4 and IL-13 (Supplemental Fig. 4a, b). The AHR and the airway inflammatory response to asperamide B required the presence of *i*NKT cells, since it failed to occur in *CD1d*<sup>−/−</sup> mice (Fig. 5i), and also required IL-4 and IL-13, since it failed to occur in *Il4*<sup>−/−</sup>*Il13*<sup>−/−</sup> mice (Fig. 5j and Supplemental Fig. 4c). These findings indicate that asperamide B is a major component of *A. fumigatus* that can induce the development of AHR and airway inflammation.

### Role of IL-33 in asperamide B-induced AHR

Like the *A. fumigatus* extract, asperamide B failed to induce AHR in *Myd88*<sup>−/−</sup> mice after a single dose (Fig. 6a). However, asperamide B required IL-33R/ST2 signaling, since *Il1rl1*<sup>−/−</sup> mice, which lack the receptor for IL-33, failed to respond to asperamide B (Fig. 6b). *Il1rl1*<sup>−/−</sup> mice showed normal populations of *i*NKT cells compared to WT mice, ruling out a deficiency in *i*NKT cells as a cause for the absence of AHR in *Il1rl1*<sup>−/−</sup> mice (Supplemental Fig. 5). We therefore hypothesized that asperamide B-induced AHR requires MyD88, because Il1Rap, a subunit of the IL-33 receptor, requires MyD88 for signaling. Indeed, IL-33 mRNA was greatly increased in the lungs of mice within 3 hrs of administration of



asperamide B (Fig. 6c). In addition, asperamide B induced IL-33 production in several cell types, including alveolar and interstitial macrophages (Fig. 6d). The induction of IL-33 in alveolar macrophages appeared to be *i*NKT cell dependent, since *i*NKT cells cultured with alveolar macrophages and asperamide B resulted in a significant increase in IL-33 mRNA in the cultures (Fig. 6e). These results indicate that *A. fumigatus* contains a glycolipid antigen that activates *i*NKT cells, which can induce alveolar macrophages and other APCs to produce IL-33, which in turn is required for the development of AHR. In addition, these results together indicate that asperamide B directly activates *i*NKT cells, and that the *in vivo* requirement for MyD88 in the AHR response to *A. fumigatus* reflects the need for ST2 signaling through the IIIIRap.

## Discussion

In these studies, we describe the identification of a fungal glycosphingolipid antigen, asperamide B, that directly activated *i*NKT cells in a CD1d-restricted, MyD88-independent manner. To our knowledge, this is the first report of a fungal glycolipid antigen that *directly* activates both mouse and human *i*NKT cells, and therefore these studies very significantly extend the range of microorganisms that are known to directly activate *i*NKT cells. Moreover, we demonstrated that purified as well as synthesized asperamide B, by activating *i*NKT cells, rapidly induced AHR, a cardinal feature of asthma, in the absence of adaptive immunity, and that asperamide B, when loaded into CD1d, could directly stain and activate *i*NKT cells. Because *A. fumigatus* is ubiquitous in the environment and is frequently recovered from the respiratory tract, our observations are clinically important and help to explain the potency of *A. fumigatus* in causing respiratory disease and severe asthma in humans.

The number of distinct microorganisms known to activate *i*NKT cells has grown rapidly over the past several years, but no previous study has found fungal glycolipid antigens that *directly* activate *i*NKT cells<sup>19</sup>. Initially, *i*NKT cells were thought to react primarily to Gram negative bacteria that lack LPS (e.g., *Sphingomonas* species)<sup>20, 21</sup>. However, subsequent studies demonstrated that *i*NKT cells recognized lipids from several additional bacterial species, including *Borrelia burgdorferi*<sup>22</sup>, *Helicobacter pylori*<sup>23</sup>, *Streptococcus pneumoniae* and Group B *Streptococcus*<sup>24</sup>. Our results showing that glycolipids from *A. fumigatus* directly activate *i*NKT cells extend the range of microorganisms that are directly detected by *i*NKT cells into another biological Kingdom, i.e., Fungi. Therefore, the ability of the semi-invariant TCR of *i*NKT to function as a pattern recognition receptor that recognizes both pathogenic bacteria and fungi may explain the extensive conservation of this *i*TCR in most mammalian species<sup>25</sup>.

Previous studies have suggested that although some microbial glycolipids can directly activate *i*NKT cells, the dominant pathway for *i*NKT cell activation during microbial infection involves the indirect pathway, in which *i*NKT cells respond secondarily to IL-12 produced by APCs activated by microbial PAMPs binding to pattern recognition receptors (PRR)<sup>26</sup>. In particular, *A. fumigatus* express  $\beta$ -1, 3 glucans<sup>18, 27, 28</sup>, which trigger dectin-1 and induce the production of IL-12, which then secondarily activates *i*NKT cells<sup>18</sup>. However, *A. fumigatus* causes several different types of pathologies, including invasive

tissue disease, but also hypersensitivity disease and asthma. While the indirect, IL-12-dependent pathway for *i*NKT cell activation may be critically important during invasive aspergillosis<sup>29</sup>, it may be less relevant for *Aspergillus* hypersensitivity disease occurring at mucosal surfaces<sup>30</sup>. In this mucosal response, asperamide B may directly activate *i*NKT cells, triggering the production of IL-13 and IL-33, independently of dectin-1 and IL-12. Thus, these distinct pathways may be differentially engaged depending on the setting (e.g., in the airways versus in the tissues), although it is possible that the  $\beta$ -glucan/dectin-1 and the asperamide B pathways may overlap and synergize in some conditions, such as chronic fungal exposure<sup>31, 32</sup>.

The *A. fumigatus* glycolipid identified in our studies, asperamide B, which could activate both mouse and human *i*NKT cells, stimulated mouse *i*NKT cells *in vitro* in a MyD88-independent manner. In contrast, the *in vivo* induction of AHR by the *A. fumigatus* glycolipid required MyD88, presumably because the induction of AHR by asperamide B also required IL-33 and its receptor, ST2. Signaling through ST2 requires MyD88, since the IL-33 receptor heterodimeric complex includes both ST2 (IL-1 receptor-like 1, IL1rl1) and the IL1Rap, which recruits and utilizes the TIR binding adaptor protein, MyD88. In the lungs, we propose that asperamide B activates *i*NKT cells, which then trigger APCs such as alveolar macrophages to produce IL-33. A similar induction of IL-33 in alveolar macrophages by *i*NKT cells was recently described with  $\alpha$ -Galactosylceramide and *Sphingomonas* glycolipids in the lungs<sup>33</sup>, and in hepatocytes by *i*NKT cells in the liver<sup>34</sup>. After being secreted in the lungs, IL-33 can activate a subset of IL-33 responsive *i*NKT cells<sup>35</sup> and type 2 innate lymphoid (ILC2) cells (also called, nuocytes or natural helper cells<sup>35-40</sup>), both of which can produce IL-13 and induce AHR.

The induction of IL-33 synthesis in macrophages by asperamide B-activated *i*NKT cells may also help to explain the robust capacity of *A. fumigatus* to induce AHR. IL-33 is an innate cytokine that has been shown to potently cause AHR in the absence of adaptive immunity<sup>33, 39, 41</sup>. Notably, since only some glycolipids that activate *i*NKT cells induce AHR, the ability of asperamide B to induce IL-33 production may be an important characteristic that distinguishes it from other glycolipids that cannot induce AHR. We hypothesize that alveolar macrophages take up only some *i*NKT cell activating glycolipids, but these allow an interaction with *i*NKT cells and the production of IL-33, which then induces AHR. This IL-33-ST2 axis may be critical for the development of AHR, and is very distinct from the indirect *i*NKT cell activation pathway, which involves IL-12 and IFN- $\gamma$ <sup>18, 27, 28</sup> and clearance of infection. Thus, the specific glycolipid and APC with its associated cytokines together shape the response after *i*NKT cell activation, and may help to explain how *i*NKT cells selectively regulate distinct forms of immunity.

Moreover, because *A. fumigatus* is ubiquitous in the environment, respiratory exposure to *A. fumigatus* is common, and recognition of asperamide B by *i*NKT cells in some individuals may critically translate *A. fumigatus* exposure into significant respiratory disease. Although only 2-3% of individuals in the general population develop allergic sensitization to *A. fumigatus* (as determined by immediate skin test positivity)<sup>42, 43</sup>, in asthma, approximately 28% of patients including 45% of patients with severe asthma, develop *A. fumigatus* sensitization<sup>9, 44</sup>. The high rate of sensitization to *A. fumigatus* in severe chronic asthma



may be due to the germination in the lungs of the spores, and exposure and sensitization to the hyphae, which contain the asperamide B lipid. We therefore propose that the direct activation of *i*NKT cells by asperamide B from *A. fumigatus* at the mucosal surface in the lungs may drive the development of allergic sensitization to *Aspergillus* and to AHR, particularly in patients with severe asthma<sup>11, 45</sup>.

Although *i*NKT cells may have evolved to rapidly respond to pathogenic microorganisms, their capacity when activated to serve as an adjuvant for Th2-type immunity has been previously suggested, for example, in response to environmental glycolipids from plant pollens<sup>46</sup>, in house dust<sup>47</sup> and cow's milk<sup>48, 49</sup>. Thus, in patients with allergic disease and asthma, it is possible that glycolipids from *A. fumigatus*, cypress tree pollen, cow's milk and *Sphingomonas* species (which are often found in the lungs of patients with poorly controlled asthma<sup>50</sup>) all activate *i*NKT cells, which then provide potent adjuvant activity to enhance allergic, Th2 adaptive sensitization to accompanying proteins. The environment may thus be replete with such *i*NKT cell activating compounds that are recognized and sensed by *i*NKT cells and that may enhance allergic sensitization<sup>51</sup>. As such, *i*NKT cells, which may be increased in number in the mucosa of allergy prone children<sup>52</sup>, may play a critical role in allergy and allergic asthma. This idea is also consistent with the observation that most protein allergens have lipid binding activity<sup>53, 54</sup>, which may allow glycolipids that activate *i*NKT cells to associate with these clinically important protein allergens, facilitating Th2 sensitization.

## Methods

### Animals

Wild-type BALB/cByJ, C57Bl/6J, *H2.Ab1-Ea*<sup>-/-</sup>, and *Lps2/Lps2* were obtained from Jackson Laboratory, Bar Harbor, ME. Dr. M. Grusby, Harvard School of Public Health, provided CD1d1<sup>-/-</sup> mice, backcrossed to the BALB/c strain. Drs. M. Taniguchi, and T. Nakayama, Chiba University generously provided *Tcra-J18*<sup>-/-</sup> mice. The *Myd88*<sup>-/-</sup> strain was generated by S. Akira, and backcrossed to the BALB/c strain by Dr. R. Geha, Boston Children's Hospital, who provided breeding pairs to us. Dr. A. McKenzie, Medical Research Council, Laboratory of Molecular Biology, Cambridge, U.K, generated the *Il4*<sup>-/-</sup>*Il13*<sup>-/-</sup> and *Il1rl1*<sup>-/-</sup> mice. Female mice were studied at approximately 6-8 weeks of age and were age matched. The Animal Care and Use Committee, Children's Hospital Boston approved all animal protocols.

### *Aspergillus fumigatus* administration and airway hyperreactivity

An *Aspergillus fumigatus* fungal extract was obtained from the NIH research reagent program. Mice lightly anesthetized with isoflurane were given 100 µg of *A. fumigatus* by the intranasal route on three consecutive days. In some experiments only one dose of the *A. fumigatus* extract was given. In other experiments, purified or synthesized glycolipids suspended in vehicle (containing BSA, Tween20 and histadine) were administered intranasally to mice. One day after the final dose, mice were sedated, tracheostomized, and ventilated. Direct measurement of airway resistance and dynamic compliance was performed by invasive plethysmography (BUXCO systems). Two pressure sensors measure

change in lung volume and change in tracheal pressure. From these measurements, average lung resistance was calculated for three minutes per dose of methacholine<sup>55</sup>. After measurement of airway hyperreactivity, bronchoalveolar lavage was performed by flushing the airways twice with 1 ml phosphate buffered saline. Cells were then counted on a standard hemocytometer and centrifuged onto glass slides. Bronchoalveolar lavage cells were then stained by DiffQuick, a modified Mae-Wright-Giemsa stain. Numbers of macrophages, eosinophils, neutrophils, and lymphocytes were assessed by cell morphology.

### Flow cytometry

Alternatively, *i*NKT cells from the bronchoalveolar lavage were stained with the following antibodies/reagents CD11b-FITC, CD19-FITC, PBS57 loaded or control unloaded CD1d tetramers-PE (NIH, NIAID MHC tetramer core facility), CD45-PE-Texas Red, TCR $\beta$ -eFluor780, CD4-Alexa700. Macrophages were identified using CD45-PE-Texas Red, F4/80-FITC, and CD11c-eFluor780. All samples were blocked with 1  $\mu$ g Fc block for 15 minutes prior to antibody/tetramer staining at 4°C for 30 minutes in phosphate buffered saline containing 2% fetal calf serum (2% FCS-PBS). Cells were washed twice in 2% FCS-PBS and then data was collected on a FACSCanto (BD Biosciences). Analysis of FACS plots was performed using FlowJo (Treestar).

For intracellular staining, after fixation and permeabilization (Cytofix/Cytoperm kit; BD Biosciences), *i*NKT cells were incubated with anti-IL-13-APC and anti-IL-4-FITC, while macrophages were incubated with anti-IL-33-PE. The respective isotype control was used for each experiment.

### CD1d tetramer loading

To generate sAspB loaded mCD1d monomers, 30  $\times$  molar excess of sAspB in DMSO at 5 mg/ml was incubated with biotinylated-mCD1d (from the NIH Tetramer facility) in 4 mM CHAPS and 20 mM Tris pH7.0 overnight at room temperature with slight agitation. The mCD1d monomers were tetramerized by adding SA-PE (S868, Invitrogen) to the lipid-loaded monomers as previously described<sup>56</sup>.

### Plate bound CD1d tetramer assay

Streptavidin plates (96-well) were incubated with 0.5  $\mu$ g of CD1d monomer overnight. Plates were washed with PBS and incubated overnight with lipid in CHAPS/Tris buffer (see tetramer loading). Plates were washed with PBS and then cultured with  $5 \times 10^5$  *i*NKT cells for 48hrs. In some wells, 5  $\mu$ g/ml anti-CD28 mAb was added. Supernatants were assayed for IL-4 and IFN- $\gamma$  by ELISA.

### *Aspergillus* glycolipid antigen purification

*Aspergillus niger* and *Aspergillus fumigatus* strains were grown in growth medium. Mycelium was collected and lyophilized. Equal dry weight of both the strains were collected and extracted in similar manner. Folch extraction was performed and LC-MS was carried out on Folch's lower layer. *Aspergillus fumigatus* mycelia were extracted in 2:1 CHCl<sub>3</sub>:MeOH three times. All of the extracts were pooled and dried. Liquid-liquid extraction according to Folch's procedure was performed to remove highly polar

contaminants. The lower layer was dried and subjected to silica gel column chromatography. The crude glycolipid fraction was further purified by acylation and deacylation protocols to yield pure compound.  $^1\text{H}$  and 2D NMRs and ESI-MS/CID-MS techniques were used for structure determination. The glycolipid was identified as  $\beta$ -glycosylceramide having (4*E*, 8*E*)-9-methyl-4, 8-sphingadienine as long chain base and *N*-2'-hydroxy-(*E*)-3'-octadecenoate as an acid moiety (Chaudhary et al, manuscript in preparation).

### Quantitative PCR

To measure RNA expression, lung tissue was pooled and homogenized in TRIzol reagent and total lung RNA was purified according to the manufacturer's instructions. Quantitative PCR was performed on an ABI7300 using Applied Biosystem primers and probes for the indicated genes in triplicate wells. Gene expression was normalized using GAPDH. Delta cycle time (d-ct) for the gene of interest was calculated by subtracting the ct of GAPDH from the ct of the gene of interest. dd-ct was calculated by subtracting the treated d-ct from the control d-ct. Fold change is calculated as  $2^{\text{dd-ct}}$ . Standard deviation for dd-ct was calculated using the standard square root of squares method. Error bars in the figure are calculated as  $2^{\text{dd-ct} + \text{s.d.}(\text{dd-ct})}$ . Statistical analysis was calculated on normalized d-ct values between treated and control groups with an n=3.

### iNKT cell lines

Mouse and human iNKT cells lines were generated and maintained in our laboratory as previously described<sup>39, 57</sup>. iNKT cells ( $5 \times 10^4$ ) were cocultured with GM-CSF treated bone marrow derived DCs ( $10^4$ ) and the indicated glycolipids for 48 hrs. Supernatants were assessed for IL-4 and IFN- $\gamma$  by ELISA.

### Statistical tests

Data from *in vivo* experiments are presented as mean  $\pm$  s.e.m., while data from *in vitro* experiments (iNKT/BMDC cocultures) are presented as mean  $\pm$  s.d. Data were analyzed by ANOVA or unpaired student's t test using Prism 4 (GraphPad Software Inc.).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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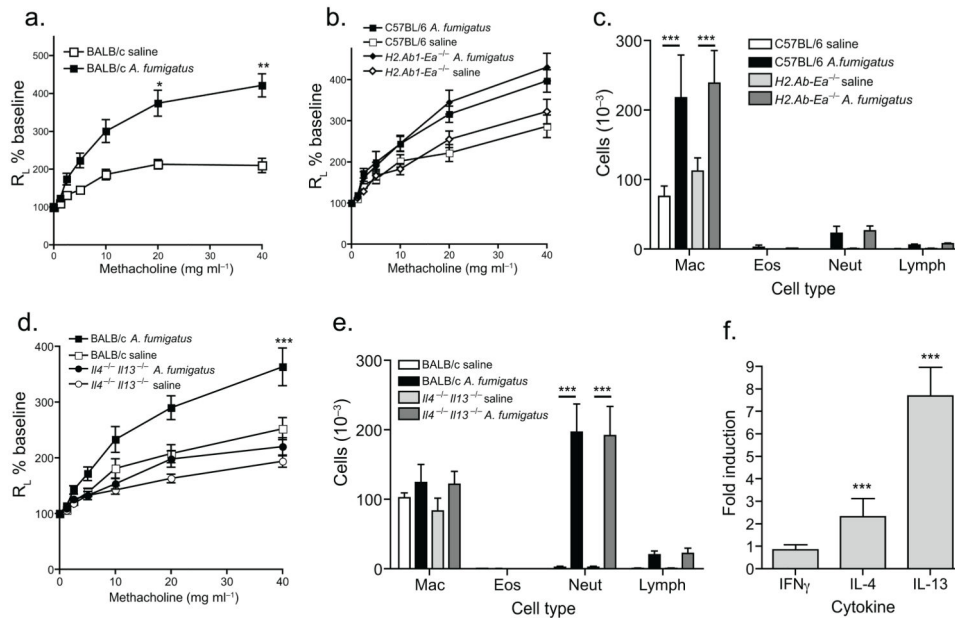
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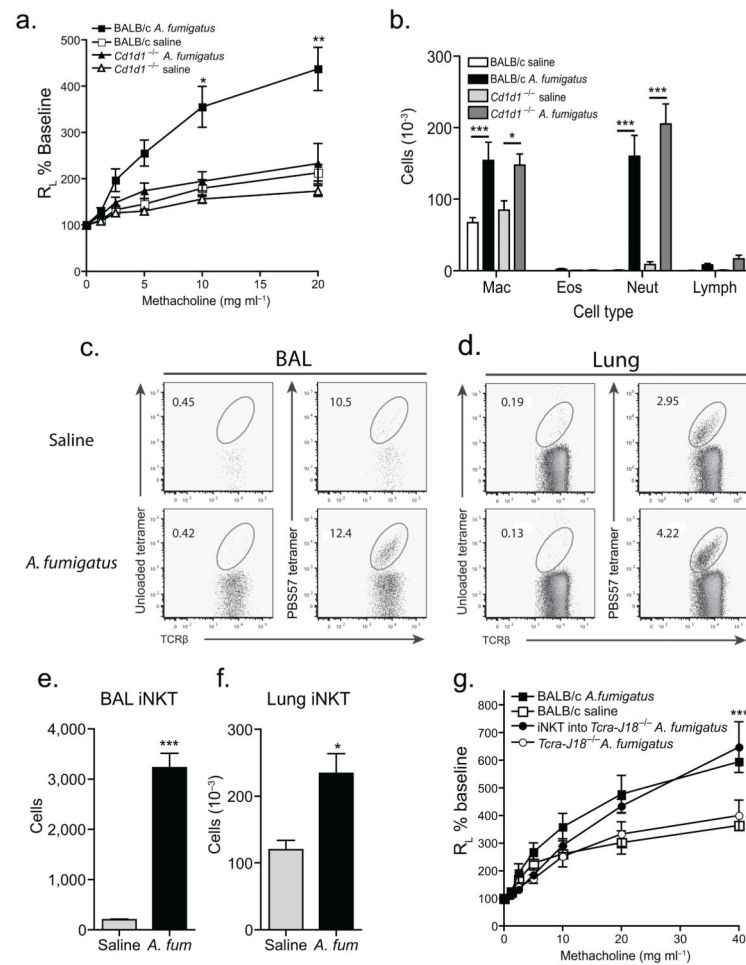
## Abbreviations

<b>AHR</b>	airway hyperreactivity
<b>APC</b>	antigen presenting cells
<b>BAL</b>	bronchoalveolar lavage
<b>BMDC</b>	bone marrow derived dendritic cells
<b>IL1Rap</b>	IL-1 receptor accessory protein
<b>NKT</b>	Natural Killer T
<b>PAMP</b>	pathogen associated molecular pattern
<b>WT</b>	wild-type



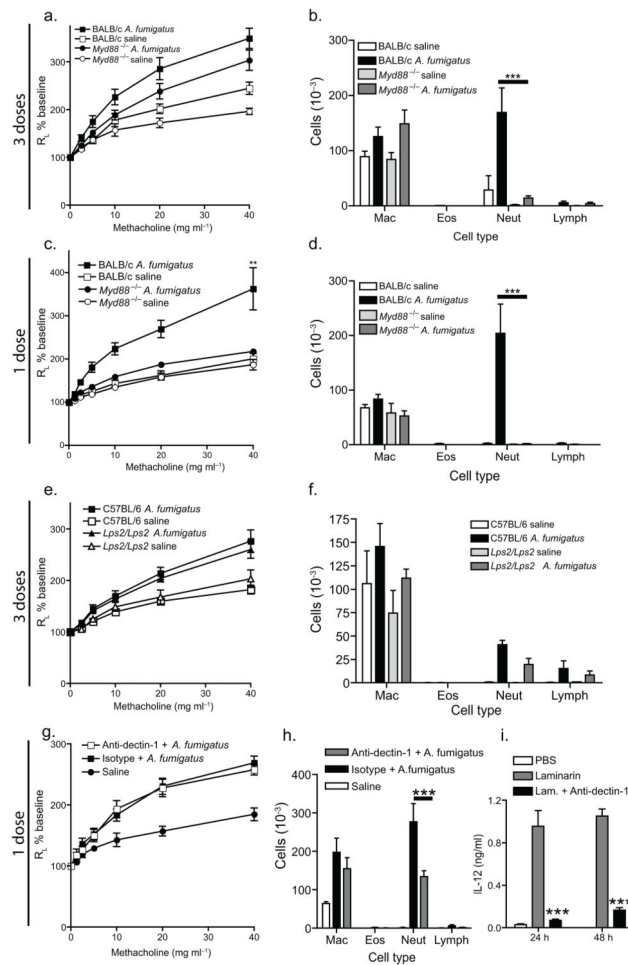


**Figure 1. An *A. fumigatus* extract rapidly induces AHR independent of adaptive immunity (a-e).** Measurement of airway hyperreactivity (AHR) and differential cell count of bronchoalveolar lavage (BAL) after treatment with three 100  $\mu$ g intranasal doses of an *A. fumigatus* extract. (mean  $\pm$  s.e.m., \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  Two-Way ANOVA, Bonferoni post-test). (a) AHR of wild-type BALB/c mice. Pooled data of three experiments are shown (saline  $n = 11$ , A.f.  $n = 13$ ). (b) AHR and (c) BAL count of mice deficient for MHC class II (*H2.Ab1-Ea*<sup>-/-</sup>) and C57BL/6 mice as control. Pooled data of two experiments are shown (WT  $n = 5$ , MHCII<sup>-/-</sup> Saline  $n = 7$ , A.f.  $n = 8$ ). (d) AHR and (e) BAL count of *Il4*<sup>-/-</sup>*Il13*<sup>-/-</sup> double knockout mice and BALB/c as control. Pooled data of three experiments are shown. ( $n = 9$ ). (f) Quantification of mRNA expression by QPCR in the lungs of *A. fumigatus* treated mice. Fold induction of *A. fumigatus* treated over saline treated mice is shown (mean  $\pm$  s.d., \*\*\*  $p < 0.001$ , Two-way ANOVA, Bonferoni post-test) One representative experiment of five is shown.

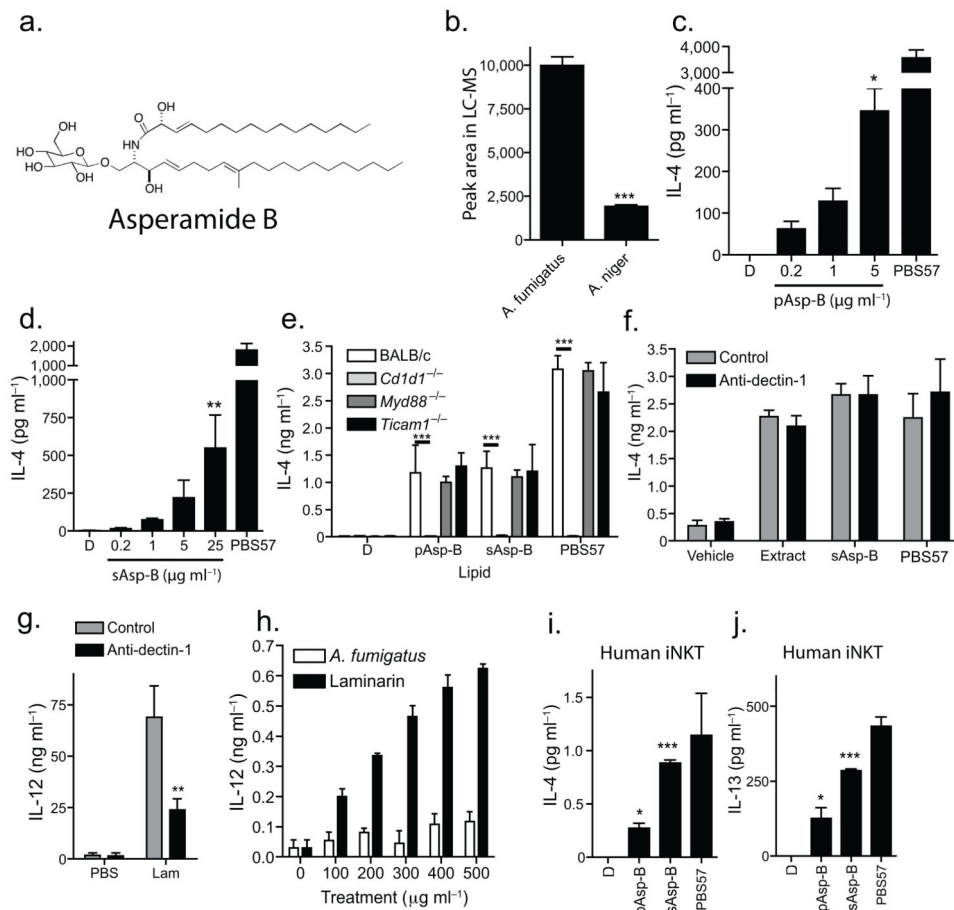


**Figure 2. AHR induced by *A. fumigatus* extract is dependent on iNKT cells**

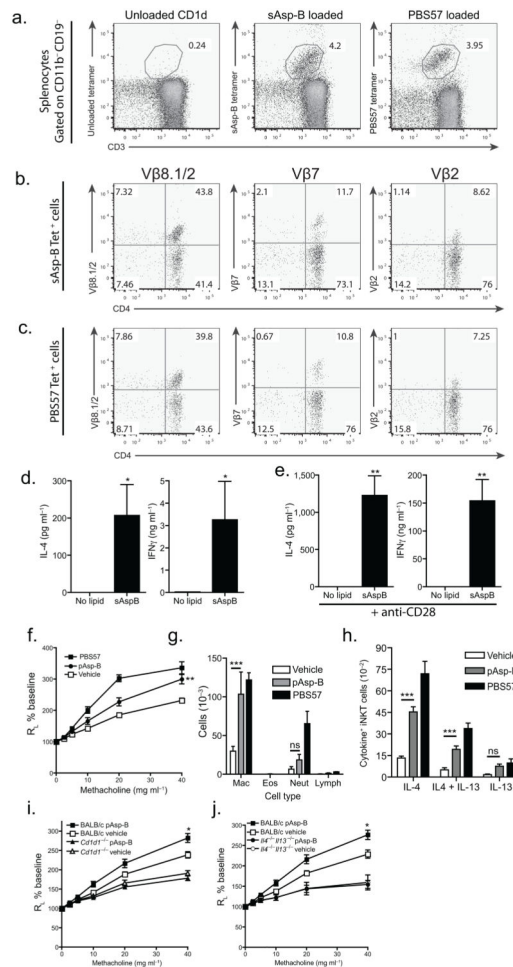
(a) Measurement of AHR and (b) BAL cell count in *CD1d1*<sup>-/-</sup> and BALB/c treated with three intranasal 100 µg doses of *A. fumigatus* extract. Pooled data from two experiments are shown (WT n = 7, *CD1d1*<sup>-/-</sup> n = 6), (mean ± s.e.m., \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, Two-Way ANOVA, Bonferoni post-test). (c-f) (c) BAL or (d) lung cells from saline or *A. fumigatus* treated mice were analyzed by FACS for iNKT cells. PBS57 (α-GalCer analogue) loaded CD1d tetramer and TCRβ staining identify iNKT cells with unloaded tetramer as a control. Number shows % of CD45<sup>+</sup> lymphocytes falling within the iNKT gate. One representative mouse of three is shown. The absolute number of iNKT in the (e) BAL or (f) lung is shown (mean ± s.e.m., \* p < 0.05, \*\*\* p < 0.001, student's t-test). (g) AHR of *Tcrα.J18*<sup>-/-</sup> mice that received 10<sup>6</sup> PBS57-CD1d tetramer sorted iNKT cells from the spleen of wild-type mice treated with intranasal *A. fumigatus* extract. One representative experiment of three is shown (n=4, \*\*\* p < 0.001 Two-Way ANOVA, Bonferoni post-test).



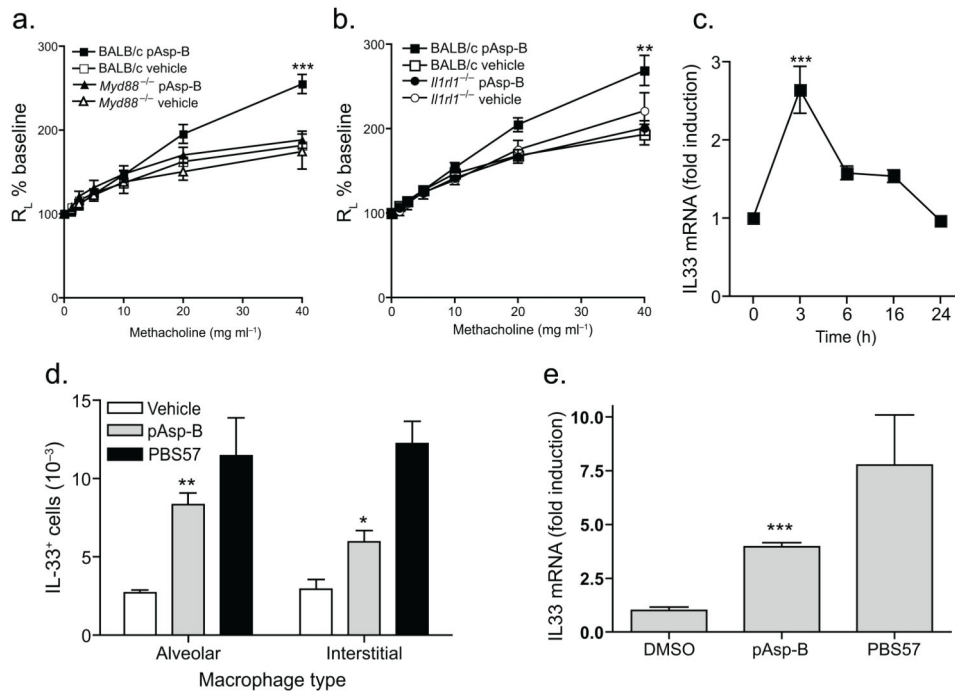
**Figure 3. AHR induced with *A. fumigatus* extract is dependent on MyD88 but not Ticam1 (a-h).** Measurement of airway hyperreactivity (AHR) and differential cell count of bronchoalveolar lavage (BAL) after treatment with the indicated number of 100  $\mu$ g intranasal doses of an *A. fumigatus* extract. (mean  $\pm$  s.e.m., \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  Two-Way ANOVA, Bonferroni post-test). (a) AHR and (b) BAL count of *Myd88*<sup>-/-</sup> and BALB/c mice administered three doses. Pooled data from three experiments is shown (WT  $n=9$ , *Myd88*<sup>-/-</sup> saline  $n=8$ , A.f.  $n=10$ ). (c) AHR and (d) BAL count of *Myd88*<sup>-/-</sup> and BALB/c mice administered one dose. Representative data from one of two experiments are shown ( $n=4$ ). (e) AHR and (f) BAL count of mice deficient for Ticam1 (*Lps2/Lps2*) and C57BL/6 mice as control. Pooled data from two experiments is shown (WT  $n=7$ , *Lps2*  $n=6$ ). (g) AHR and (h) BAL count of BALB/c mice that were administered an antibody to Dectin-1 or isotype control (500  $\mu$ g/mouse), and intranasal *A. fumigatus* extract ( $n=5$ ). (i) Measurement of serum IL-12 by ELISA after treatment with 100 mg Laminarin. An antibody to Dectin-1 or isotype control (500  $\mu$ g/mouse) was administered 12 h prior (\*\*\*  $p < 0.001$ , student's t-test).



**Figure 4. Purified and synthetic asperamide B activate mouse and human iNKT cells**  
**(a)** Structure of isolated *Aspergillus fumigatus* lipid, asperamide B, with a molecular weight of 738. **(b)** Asperamide B peak area in a liquid chromatography-mass spectrometry chromatogram is shown for *A. fumigatus* and *A. niger* (\*\*\* p < 0.001, student's t-test). **(c-f)** Measurement of IL-4 at 48 hours in coculture supernatants of  $5 \times 10^4$  iNKT and  $10^4$  BMDC treated with the indicated lipids (mean  $\pm$  s.d.). **(c)** Cocultures treated with DMSO (D), purified asperamide B (pAsp-B), or PBS57 ( $\alpha$ -GalCer analogue). **(d)** Cocultures treated with DMSO (D), synthetic asperamide B (sAsp-B), and PBS57. **(c-d)** \* p < 0.05, \*\* p < 0.01, One-Way ANOVA, Dunnett's Multiple Comparison Test to DMSO Control. **(e)** Cocultures with BMDC from BALB/c, *CD1d1*<sup>-/-</sup>, *Myd88*<sup>-/-</sup> or *Ticam1*<sup>-/-</sup> treated with the indicated lipids (\*\* p < 0.01, \*\*\* p < 0.001, Two-Way ANOVA, Bonferoni post-test). **(f)** Cocultures were incubated with an antibody specific for Dectin-1 ( $2 \mu\text{g ml}^{-1}$ ) and treated with vehicle, *A. fumigatus* extract, sAsp-B, or PBS57. **(g)** Measurement of IL-12 by ELISA at 24 hours in BMDC cultures treated with laminarin ( $500 \mu\text{g ml}^{-1}$ ) with or without anti-dectin-1 mAb ( $2 \mu\text{g ml}^{-1}$ ), (\*\* p < 0.01, student's t-test). **(h)** Measurement of IL-12 by ELISA at 24 hours in BMDC cultures treated with *A. fumigatus* extract or laminarin at the indicated concentrations. **(i-j)** Measurement of **(i)** IL-4 and **(j)** IL-13 by ELISA at 48 hours in cocultures of primary human iNKT cell lines and APCs treated with DMSO, pAspB, sAspB, or PBS57. (\* p < 0.05, \*\*\* p < 0.001, student's t-test compared to DMSO control) **(a-j)** mean  $\pm$  s.d.



**Figure 5. Asperamide B loaded CD1d stains and directly activates iNKT cells**  
**(a)** FACS plots showing spleen cells stained for CD3 and CD1d tetramers loaded with vehicle (left panel), sAsp-B (middle panel) or PBS57 (right panel). **(b-c)** FACS plots showing CD4 and Vβ usage by **(b)** sAsp-B tetramer<sup>+</sup> cells and **(c)** PBS57 tetramer<sup>+</sup> cells. **(d)** Cytokine production from iNKT cells cultured on plates coated with CD1d monomers loaded with sAsp-B (300 μg ml<sup>-1</sup>) or no lipid. **(e)** iNKT stimulated with plate bound CD1d loaded with sAsp-B plus 5 μg ml<sup>-1</sup> CD28 antibody. **(d-e)** \*  $p < 0.05$ , \*\*  $p < 0.01$ , student's t-test. **(f)** Measurement of AHR and **(g)** BAL cell count in BALB/c treated with a single intranasal dose of vehicle (n = 9), 5 μg of pAsp-B (n = 8), or 1 μg of PBS57 (n = 9). Pooled data from three experiments are shown. **(h)** Measurement of IL-4 and IL-13 secreting by iNKT cells in mice treated with vehicle, pAsp-B, or PBS57. **(f-h)** mean ± s.e.m., ns  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Two-Way ANOVA, Bonferoni post-test **(i-j)** Measurement of AHR in **(i)** CD1d1<sup>-/-</sup> or **(j)** IL4<sup>-/-</sup>IL13<sup>-/-</sup> mice treated with 5 μg of pAsp-B or vehicle. BALB/c control is the same in i and j, (WT n = 19, CD1d1<sup>-/-</sup> n = 7, IL4<sup>-/-</sup>IL13<sup>-/-</sup> n = 6). Pooled data from four experiments are shown.



**Figure 6. Asperamide B-induced AHR requires MyD88 and ST2**

(a-b) Measurement of AHR after treatment with a single intranasal dose of 5  $\mu$ g of pAsp-B or vehicle (mean  $\pm$  s.e.m., \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , Two-Way ANOVA, Bonferroni post-test). (a) AHR measured in *Myd88*<sup>-/-</sup> and BALB/c control mice (WT  $n = 4$ , *Myd88*<sup>-/-</sup>  $n = 5$ ) treated with pAsp-B. Representative data from one of two experiments are shown. (b) AHR measured in *Il1rl1*<sup>-/-</sup> (ST2) and BALB/c control mice (WT  $n = 7$ , *Il1rl1*<sup>-/-</sup>  $n = 6$ ). Pooled data of two experiments are shown. (c) IL-33 mRNA measured by QPCR from mice treated with pAsp-B. Fold induction of IL-33 mRNA compared to the zero time-point is shown. One representative experiment of two is shown (mean  $\pm$  s.e.m., \*\*\*  $p < 0.001$  One-Way ANOVA, Tukey's multiple comparison post-test). (d) The number of IL-33<sup>+</sup> lung macrophages following treatment with vehicle, 5  $\mu$ g of pAsp-B, or 1  $\mu$ g of PBS57. Alveolar macrophages were defined as F4/80<sup>+</sup>CD11c<sup>+</sup> and interstitial macrophages were defined as F4/80<sup>+</sup>CD11c<sup>-</sup>. One representative experiment of two is shown (mean  $\pm$  s.e.m., \*  $p < 0.05$ , \*\*  $p < 0.01$ , Two-Way ANOVA, Bonferroni post-test). (e) IL-33 mRNA was measured by QPCR after 24 hours from cocultures of *i*NKT cells with alveolar macrophages incubated with DMSO, pAsp-B, or PBS57 (mean  $\pm$  s.d., \*\*\*  $p < 0.001$ , student's t-test).